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Evaluation of immunoassays for the measurement of erythropoietin (EPO) as an indirect biomarker of recombinant human EPO misuse in sport

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Abstract

The measurement of serum erythropoietin (EPO) has been proposed as one of the indirect biomarkers for the detection of recombinant human EPO misuse in sport. An extended inter-laboratory validation of two commercial immunoassays for EPO measurement is described. A chemiluminescent immunoassay kit (CHEM) and an enzyme-linked immunosorbent assay kit (ELISA) were evaluated.

The CHEM assay showed intra-laboratory precision better than 6% and correct accuracy values for all quality control samples tested. Precisions and accuracies better than 7 and 13%, respectively, were obtained for the ELISA assay for most of the quality control samples. The limit of quantification estimated for CHEM assay was lower than for the ELISA assay.

Inter-laboratory concordance was good for both the assays, with lower dispersion shown by the CHEM assay. Results obtained with the ELISA assay were always lower than those of the CHEM assay. However, a good inter-technique correlation was obtained ($[\text{ELISA}] = 0.76\,[\text{CHEM}] + 0.06$, $r^2 = 0.92$).

Quality control samples had a good stability after one and two freeze/thaw cycles and in simulated transportation conditions.

In conclusion, CHEM and ELISA assays showed similar characteristics regarding intra-laboratory validation. Better inter-laboratory results were obtained with the CHEM assay and, hence, it is considered the recommended assay for anti-doping control analysis.

Keywords: EPO; Indirect biomarker; Doping; Validation; Immunoassay; Stability

1. Introduction

The administration of recombinant human erythropoietin (rhEPO) produces an increase of the red cell

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mass, haemoglobin concentration and the maximal aerobic power, thus inducing an improvement of exercise performance [1–3]. There is evidence that rhEPO is misused by elite athletes to enhance sport performance [4].

The International Olympic Committee (IOC), the World Anti-Doping Agency (WADA) as well as major sports authorities have banned the use of EPO by athletes [5]. Different methods to detect rhEPO misuse have been described including direct measurement in urine [6,7], and indirect measurements based on blood markers of altered erythropoiesis [8–11].

The direct identification method is based on the analysis by isoelectric focusing, double blotting and chemiluminescent detection of the erythropoietin (EPO) present in urine. Both exogenous rhEPO and endogenous EPO, although having identical amino acid sequences, have a different glycosilation pattern giving different isoelectric profile [6,7]. However, the application of that method is limited by the time and workload required to conduct the assay. The major drawback of the urine-based tests is the disappearance of measurable levels of rhEPO from the urine soon after administration [12], despite the athlete can retain the physiologic benefits associated with an elevated red cell mass for a longer time [13,14]. EPO concentrations in urine return to base line values 4 days after the last subcutaneous rhEPO administration [13]; moreover, after 7 days from the last subcutaneous rhEPO administration, isoelectric focusing method only detects rhEPO in approximately one-half of the administrated subjects [14].

Regarding indirect biomarkers, mathematical models have been developed to indicate current (ON models) or recently discontinued (OFF models) rhEPO administration [8–11]. Those models have been refined in order to reduce the number of markers monitored while keeping their discriminating power and, very importantly, selecting those more robust and having well characterised international standards. At present, the proposed models are based on combinations of the following markers: percent reticulocytes and haemoglobin, serum soluble transferring receptor (sTfR) and total serum EPO (endogenous plus recombinant EPO) concentrations.

Regardless the model chosen, precise and accurate measurement of sTfR and EPO are needed. Measurements have to be made using techniques giving consistent results in both inter-laboratory and inter-techniques. Since serum EPO and sTfR are usually measured using immunoassays and many of them are available, studies are necessary in order to characterise their behaviour among them and between laboratories [15].

Different immunoassays exist for measuring EPO [16–20]. In this paper, we report an extended evaluation of the two immunoassays for EPO measurement that were used to develop the mathematical models to detect rhEPO misuse [8,9,11], together with an study on the stability of EPO subjected to freezing/thawing cycles and simulated storage/transportation conditions.

2. Materials and methods

2.1. Immunological techniques

Two immunoassays, a chemiluminescent immunoassay and an enzyme-linked immunosorbent assay (ELISA), were evaluated for EPO measurement.

The chemiluminescent immunoassay was the Immulite EPO from Diagnostic Products Corporation (DPC, Los Angeles, CA, USA) (referred as CHEM). The procedure was applied using the automated analyser Immulite® chemiluminescent immunoassay system (DPC). A volume of 250 μl of serum was required. The Immulite system uses stored master curves generated by DPC for each internal batch of reagents. Each new batch has to be calibrated before its use using two adjustor points (low and high) supplied by the manufacturer. The adjustor points were analysed in quadruplicate. Quality control (QC) samples supplied by the manufacturer were also used (see Table 1).

The ELISA assay was the Quantikine Human EPO Immunoassay from R&D Systems (Minneapolis, USA) (referred as ELISA). A volume of 100 μl serum sample was analysed according to the instructions of the manufacturer. The absorbance at 450 nm was measured, taken 565 or 570 nm as reference wavelength using a microplate reader (Multiskan MS, Labsystems, Vantaa, Finland, in laboratory 1; and Novapath TM, Biorad, Milan, Italy, in laboratory 2). Calibration samples, supplied by the manufacturer were analysed always in duplicate at the following concentrations: 0, 2.5, 5, 20, 50, 100 and 200 mIU/ml. The QC samples

Table 1
Validation parameters of CHEM assay obtained in laboratories 1 and 2

QC	mIU/ml[a]	Assay	Intra-assay					Inter-assay				
			n	Mean (mIU/ml)	S.D. (mIU/ml)	Precision[b] R.S.D. (%)	Accuracy[c] error (%)	*n*	Mean (mIU/ml)	S.D. (mIU/ml)	Precision[b] R.S.D. (%)	Accuracy[c] error (%)
Laboratory 1												
1	13–17.2	1	5	14.1	0.7	5.3	Correct	14	14.2	0.5	3.6	correct
		2	5	14.5	0.4	2.8	Correct					
		3	4	14.1	0.2	1.6	Correct					
2	25.2–32.8	1	5	27.3	0.2	0.7	Correct	15	26.8	0.8	3.1	correct
		2	5	27.0	0.5	1.8	Correct					
		3	5	26.2	1.1	4.4	Correct					
3	48.5–66.5	1	5	53.4	1.6	3.1	Correct	15	51.8	2.5	4.7	correct
		2	5	51.0	2.6	5.1	Correct					
		3	5	51.1	2.7	5.2	Correct					
Laboratory 2												
1	11.7–16.5	1	4	14.2	0.4	2.5	Correct	13	13.8	0.6	4.3	correct
		2	4	14.2	0.3	2.2	Correct					
		3	5	13.1	0.2	1.8	Correct					
2	23.3–31.5	1	4	26.9	1.0	3.7	Correct	12	26.1	1.1	4.1	correct
		2	3	26.7	0.2	0.6	Correct					
		3	5	25.1	0.4	1.8	Correct					
3	45.4–61.4	1	4	51.2	1.4	2.7	Correct	13	51.2	2.0	3.9	correct
		2	4	53.2	0.6	1.1	Correct					
		3	5	49.5	1.6	3.3	Correct					

LOD: 0.2 mIU/ml; LOQ: 0.5 mIU/ml; measure range up to 200 mIU/ml.
[a] Acceptance concentration range according to the manufacturers.
[b] Measured as relative standard deviation (R.S.D.).
[c] Correct: inside the acceptance range defined by the manufacturer.

were prepared by dilution of the EPO 200 mIU/ml calibration sample with the specimen diluent (both supplied by the manufacturer) to a final concentration of 4 mIU/ml (QC 4), and 10 mIU/ml (QC 5).

2.2. Serum samples

A group of 112 healthy subjects (91 males and 21 females) aged between 18 and 55 years (mean ± standard deviation, 30 ± 9 years) took part in the study. The subjects were untrained individuals and not competing athletes. An informed consent was signed by all the subjects. No illness or medications known to impair exercise or to alter endocrine function were declared by any of those individuals. The study was approved by the local ethics committee (CEIC/IMAS no. 2000/1145/I). Confidentiality aspects of samples and results were guaranteed.

Venous blood samples were collected from each volunteer from the antecubital vein and, after an interval for clotting, they were centrifuged. Serum samples were stored at −80 °C until analysis.

2.3. Validation assays

Validation assays were performed in two independent laboratories: Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica, Barcelona, Spain (laboratory 1), and Drug Research and Control Department, Istituto Superiore di Sanità, Rome, Italy (laboratory 2). The following studies were performed.

2.3.1. Intra-laboratory validation

Intra-laboratory validation for CHEM and ELISA techniques was performed in both laboratories during four consecutive days.

For the ELISA assay, the parameters for the best fit between signal and concentration were calculated according to the mathematical model proposed by the manufacturer (absorbance versus logarithm of EPO concentration). As a measure of the goodness of fit, the error (%) in the back-calculated concentration of the calibration samples was monitored.

Up to five replicates of two or three QC samples were analysed for the determination of intra-assay precision and accuracy, while the inter-assay precision and accuracy were determined for all values obtained along three independent experimental assays of the aforementioned QC samples. Precision was expressed as the relative standard deviation (R.S.D.%) of the measurements performed. For ELISA assay, accuracy was expressed as the relative error (%) of the value obtained with respect to the assigned value for the QC samples 4 and 5. For CHEM assay, accuracy was evaluated as "correct" or "incorrect" if the concentration obtained was inside or outside the acceptance concentration range defined by the manufacturer for QC samples 1–3.

To calculate the limits of detection (LOD) and limits of quantification (LOQ), the blank calibration sample (absence of analyte) was analysed five times in the same run. The standard deviation of the values obtained was taken as the measure of the noise. LOD and LOQ were defined as the mean value obtained for the blank sample plus three and ten times the estimated value of the noise, respectively.

2.3.2. Inter-laboratory and inter-technique validation

Inter-laboratory validation was performed by analysing human serum samples from non-athletic as well as athletic population (none having used rhEPO) in two different laboratories. Inter-technique validation was performed in laboratory 1, by analysing human serum samples using both assays.

The intra-class correlation coefficient (ICC) using random effects mode was calculated to evaluate the concordance of results between laboratories [21]. Inter-technique concordance was evaluated using the Passing–Bablok method [22].

To evaluate the dispersion of the results obtained between different laboratories or between different techniques, a modification of Bland–Altman plots was used [23]. The mean values of concentrations were represented versus the relative differences between concentrations. The 95% limits of agreement (95% LA) was calculated according to the following expression: relative difference mean $\pm$ 1.96 $\times$ standard deviation of relative differences.

2.3.3. Stability studies

Stability studies were performed using the QC samples prepared from the reagents supplied by the ELISA assay manufacturer (QC samples 4 and 5, Table 2).

2.3.3.1. Freeze/thaw (F/T) cycles. The stability in freeze/thaw cycles of QC samples was assessed by comparing the results of the QC samples analysed immediately after their preparation (F/T0) with those obtained after storage at $-80\,^{\circ}$C for 30 min, thawed at room temperature and analysed (F/T1) or repeating the same cycle two times (F/T2).

2.3.3.2. Storage/transportation conditions. Stability in storage/transportation conditions was evaluated in QC samples following a simulated transportation conditions protocol consisting of storing QC samples at $-80\,^{\circ}$C for 48 h (simulated storage), at $-20\,^{\circ}$C for 48 h (simulated transportation), at $-80\,^{\circ}$C for at least 48 h (simulated storage at destination), and thawed at room temperature for analysis. Results obtained were compared with those obtained for the sample analysed right after preparation.

Stability was evaluated by monitoring the percentage of the degradation of the analyte in each storage condition.

All statistical calculations were done using the statistical package SPSS 2001 for Windows, version 11.5.1 (SPSS Inc., Chicago, IL, USA).

3. Results

Validation parameters for the CHEM assay are shown in Table 1. Intra- and inter-assay precision, measured as the R.S.D., was always lower than 6%. The concentrations of the QC samples analysed were in all cases inside the acceptance range described by the manufacturer. The LOD and LOQ values estimated for CHEM assay in laboratory 1 were 0.2 and 0.5 mIU/ml, respectively.

Table 2
Validation parameters of ELISA assay obtained in laboratories 1 and 2

QC	mIU/ml	Assay	Intra-assay					Inter-assay				
			n	Mean (mIU/ml)	S.D. (mIU/ml)	Precision[a] R.S.D. (%)	Accuracy[b] error (%)	*n*	Mean (mIU/ml)	S.D. (mIU/ml)	Precision[a] R.S.D. (%)	Accuracy[b] error (%)
Laboratory 1												
4	4	1	5	3.6	0.2	6.5	10.9	15	3.5	0.2	4.7	11.8
		2	5	3.5	0.1	3.9	11.8					
		3	5	3.5	0.1	4.1	12.8					
5	10	1	5	9.3	0.2	2.3	7.0	15	9.3	0.3	3.4	7.3
		2	5	9.4	0.5	5.1	6.2					
		3	5	9.2	0.2	2.3	8.5					
Laboratory 2												
4	4	1	5	3.9	0.3	8.4	5.6	15	4.0	0.7	18.0	11.7
		2	5	4.0	1.3	30.9	22.5					
		3	5	4.1	0.4	9.2	6.9					
5	10	1	5	10.1	0.8	8.0	5.8	15	10.0	0.9	8.7	6.7
		2	5	9.6	1.0	10.7	8.6					
		3	5	10.3	0.8	7.5	5.7					

LOD: 0.6 mIU/ml; LOQ: 2.0 mIU/ml; measure range 2.5–200 mIU/ml.
[a] Measured as relative standard deviation (R.S.D.).
[b] Measured as the relative error respect the assigned QC sample value.

Intra-laboratory validation for ELISA assay is described in Table 2. The intra- and inter-assay precision for laboratory 1 were lower than 7% for both QC samples. The intra- and inter-assay accuracy was better for the QC 5 (QC with the highest concentration) but in all cases lower than 13%. For laboratory 2, the precision and accuracy results were in almost all cases of the same order than in laboratory 1. The LOD and the LOQ calculated in laboratory 1 were 0.6 and 2 mIU/ml, respectively, higher than those obtained by the CHEM assay.

Inter-laboratory results for CHEM and ELISA assays are presented in Figs. 1 and 2. High concordances were obtained for both assays (ICC = 0.980 and 0.920, respectively). The dispersion of results obtained with CHEM measured as the 95% LA was from −16.87 to 14.44% (Fig. 1). Higher dispersion was obtained for ELISA assay, from −37.42 to 15.97% (Fig. 2).

The inter-technique comparison results are shown in Fig. 3. The results obtained with the ELISA test were always lower than those obtained with the CHEM assay. Passing–Bablok method showed that CHEM and ELISA assays were not comparable (95% confidence intervals did not include the value 0 for intercept, nor value 1 for the slope). The differences in results obtained between techniques expressed as 95% LA were from −51.22 to −5.13%. However, the relation between results follows the equation: $[\text{ELISA}] = 0.76\,[\text{CHEM}] + 0.06$, with a good correlation ($r^2 = 0.92$).

In reference to freeze/thaw stability studied for QC samples 4 and 5 (Fig. 4), no relevant degradation was observed after one and two freeze/thaw cycles, with differences to initial concentration (F/T0) lower than 5% for both QC samples. Regarding the stability in simulated transportation conditions, differences to initial concentration lower than 5% were also obtained.

4. Discussion

The measurement of serum EPO concentration has been proposed as one of the serum biomarkers of rhEPO misuse in sport [8,9,11]. Moreover, EPO measurement has important clinical applications in differential diagnosis of anemia and polycythemia, and monitoring rhEPO administration in EPO deficient patients [18].

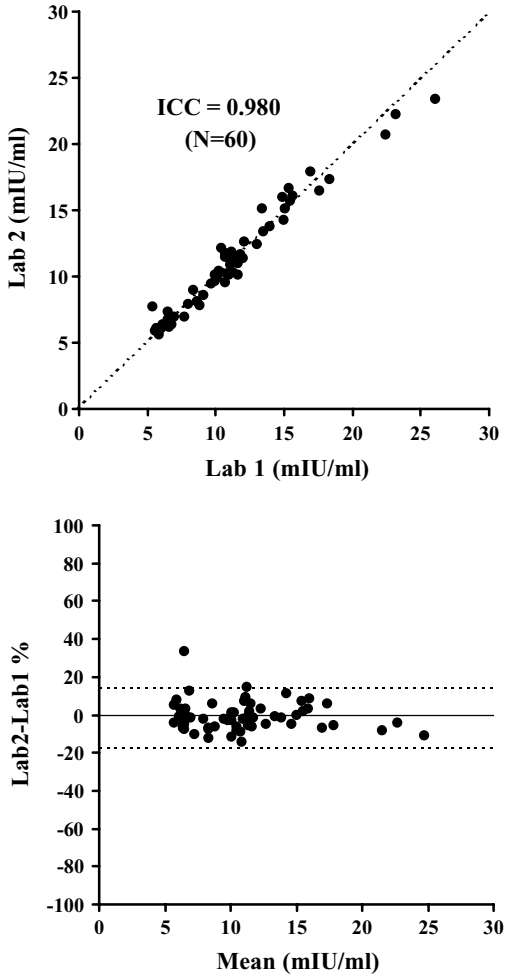

Fig. 1. Inter-laboratory comparison of EPO CHEM assay. Top: graphical comparison between laboratories and intra-class correlation coefficient (ICC), dotted lines representing total concordance. Bottom: modified Bland–Altman plots (see text), dotted lines representing the 95% limits of agreement.

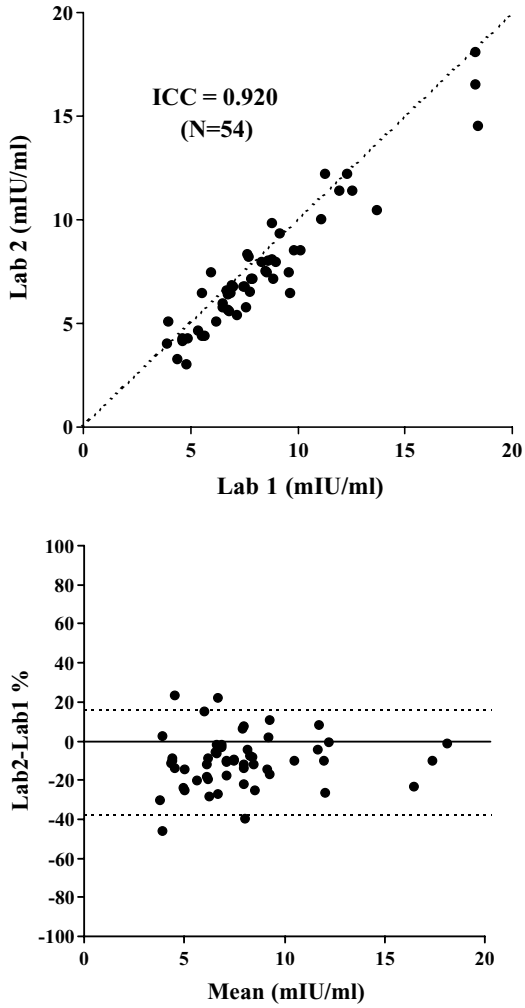

Fig. 2. Inter-laboratory comparison of EPO ELISA assay. Top: graphical comparison between laboratories and intra-class correlation coefficient (ICC), dotted lines representing total concordance. Bottom: modified Bland–Altman plots (see text), dotted lines representing the 95% limits of agreement.

Usually, concentration of EPO in serum samples is measured by using immunological techniques [16–19]. The measurement methods need intra-laboratory validation to assure satisfactory intra- and inter-assay precision and accuracy allowing clinically and analytically acceptable results. In addition, in immunological methods, the difference in specificity of the used antibodies may lead to different results when using different assays [16–19]. Thus, reference ranges for EPO concentration can vary depending on the technique used. Therefore, immunoassays require also an inter-technique validation to evaluate the concordance in the concentrations obtained using different methods. Besides, in anti-doping control analyses an homogeneous application of criteria to suspect rhEPO abuse among the different anti-doping centres is needed to assume a fair outcome for different athletes. Even sometimes, samples of the same athlete can be analysed in different laboratories,

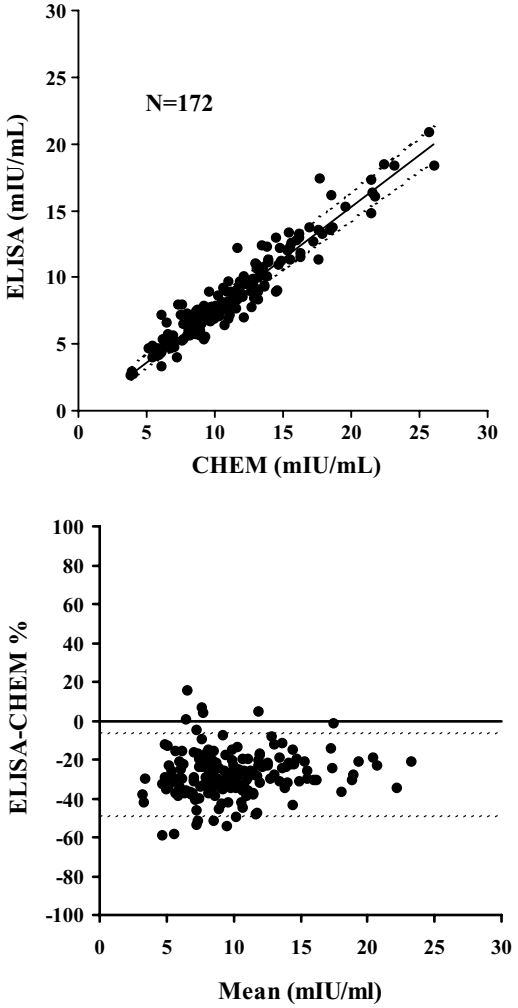

Fig. 3. Inter-technique comparison of EPO assays: CHEM and ELISA. Top: Passing–Bablok plots, dotted lines representing the 95% limits of confidence. Bottom: modified Bland–Altman plots (see text), dotted lines representing the 95% limits of agreement.

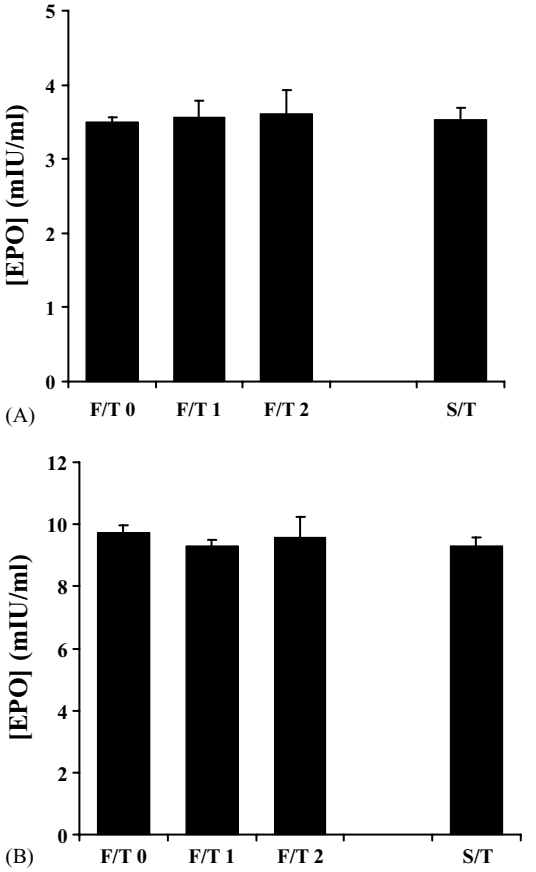

Fig. 4. Results of EPO concentrations after freeze/thaw (F/T) stability study and in simulated storage/transportation (S/T) conditions (see text): (A) QC 4 and (B) QC 5.

emphasising the need to guarantee the transferability and concordance of results between laboratories. For this reason, inter-laboratory comparisons are also needed.

In this study, two different analytical techniques have been evaluated for EPO measurement and the degree of concordance of the results obtained was also investigated. Both immunoassays were those used in previous studies of biomarkers of rhEPO misuse [8,9,11]. The main difference between the two techniques was that the CHEM assay is performed using Immulite automated analyser, which allows a reduction of the handling error as has been shown in Table 1. The two EPO assays, CHEM and ELISA, had a turnaround time for results of 1.5 and 4 h, respectively. Accordingly, in the CHEM, a shorter and easier protocol is proposed, while in ELISA more manipulations are needed.

Concentrations of QC samples of ELISA test were lower than those of CHEM test (Tables 1 and 2). The results of precision and accuracy for CHEM and ELISA tests were similar when comparing QC samples of the same level of concentration (see results of QC 1 in Table 1, and results of QC 5 in Table 2). However, the results of ELISA test were also acceptable

even for the lowest QC sample at 4 mIU/ml, located in the low limit of the serum EPO concentrations range found for the studied population (from 4 to 26 mIU/ml). The range of EPO serum concentrations studied is normal or moderately high. Higher and lower levels can be found for different pathologies or doped subjects during and after rhEPO administration [8].

The precision and accuracy obtained in the present study were similar or better than those obtained in previous studies using the same [8,9,19] or other commercially available kits for EPO measurement [16,18]. Besides, estimated LOD and LOQ for CHEM and ELISA tests were lower than most of the previously reported methods [16–18].

The results of the stability tests with EPO QC samples, prepared in a protein stabilised buffer, showed that samples were stable after two freeze/thaw cycles (−80 °C to room temperature) and also in simulated storage/transportation conditions.

Regarding inter-laboratory comparison, higher degree of concordance was obtained for the CHEM assay (Fig. 1), although results obtained with the ELISA technique appeared also acceptable (Fig. 2). The use of autoanalyser in CHEM assay would have an important role in the low differences in results obtained between both laboratories. On the other hand, the high degree of concordance in results between laboratories observed in inter-laboratory comparison showed that EPO is also stable in actual serum samples after real storage/transportation conditions. Serum samples analysed in laboratory 2 were sent from laboratory 1 using a regular courier system, i.e. packed in dry ice and arriving at destination in approximately 48 h.

Taking into account the Passing–Bablok regression method, the results obtained by both techniques are not comparable, being ELISA results lower than those obtained by the CHEM assay (Fig. 3). However, the correlation obtained between ELISA and CHEM was good and equivalent to that reported in previous studies [9]. In spite of using different calibrators (CHEM assay uses the WHO standard 67/343, natural human EPO; and ELISA assay uses the WHO standard 87/684, recombinant human EPO), the WHO standard 87/684 showed essentially identical reactivity to WHO standard 67/343 when assayed in ELISA assay (manufacturer's information). Therefore, the differences in results may not be due to the calibrators and may be explained, at least in part, by different specificity of the antibodies used.

Both studied assays (ELISA and CHEM) have been used to develop the mathematical models to detect rhEPO misuse [8,9,11], as mentioned in previous paragraphs. The CHEM assay was proposed for the initial screen and for quantitative data as it uses automated analysers that permitted a high throughput, and the ELISA tests, used in the initial studies for screening purposes, was finally considered the best choice for confirmation purposes [9]. Taking into account that EPO concentrations obtained with ELISA technique were around 25% lower than with CHEM assay, different results may be obtained depending on the assay used for screening and confirmation. In the mathematical models proposed, EPO contributes as the natural logarithm of the serum concentration [11]. A reduction of 25% in concentration may lead to a difference in the final score value of around 2 units less (ON model) or more (OFF model), which may have impact on the final evaluation of a sample with a score close to a threshold value for a given probability.

Taking into account the study performed, the CHEM technique obtained better intra- and inter-assay precision and accuracy, lower LOD and LOQ, and good inter-laboratory concordance with a low dispersion. However, this technique uses an analyser available in clinical laboratories and not usual in anti-doping control laboratories. On the other hand, ELISA test uses universally available equipment and, thus, it can be easily implemented both in clinical and anti-doping control laboratories, although it can be subjected to a higher variability due to the different technical skills of the analysts, and also subjected to inter-laboratory variation. The final choice should be dependent on the availability of equipment and the type of analysis to be performed (clinical, anti-doping control, ...). For clinical applications, where samples are analysed in one single laboratory with reference ranges obtained with the same immunological test, both assays may be suitable. However, in anti-doping control analysis, where comparability of results between laboratories is a key feature, in spite of potential use of any of both techniques for screening, it seems advisable to use the CHEM assay to release final results which can be used in inter-laboratory comparable criteria to suspect rhEPO abuse.

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